One-Dimensional SDS Gel Electrophoresis of Proteins

Electrophoresis is used to separate complex mixtures of proteins, (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates) to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in the gel matrix; pore size decreases with higher acrylamide concentrations. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

The standard Laemmli method (see Basic Protocol 1) is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). The standard method for full-size gels (e.g., 14 × 14 cm) can be adapted for the minigel format (e.g., 7.3 × 8.3 cm; see Basic Protocol 2). Minigels provide rapid separation but give lower resolution.

Several alternate protocols are provided for specific applications. The first two alternate protocols cover electrophoresis of peptides and small proteins, separations that require modification of standard buffers: either a Tris-tricine buffer system (see Alternate Protocol 1), or modified Tris buffer in the absence of urea (see Alternate Protocol 2). Continuous SDS-PAGE is a simplified method in which the same buffer is used for both gel and electrode solutions and the stacking gel is omitted (see Alternate Protocol 3). Other protocols cover the preparation and electrophoresis of various types of gels: ultrathin gels (see Alternate Protocol 4), multiple single-concentration gels (see Support Protocol 1), gradient gels (see Alternate Protocol 5), multiple gradient gels (see Support Protocol 2), and multiple gradient minigels (see Support Protocol 3). Proteins separated on gels can be subsequently analyzed by immunoblotting (*UNIT 10.8*), autoradiography or phosphor imaging (*APPENDIX 3A*), or staining with protein dyes (*UNIT 10.6*).

CAUTION: Before any protocols are used, it is extremely important to read the following section about electricity and electrophoresis.

ELECTRICITY AND ELECTROPHORESIS

Many researchers are poorly informed concerning the electrical parameters of running a gel. It is important to note that the voltages and currents used during electrophoresis are dangerous and potentially lethal. Thus, safety should be an overriding concern. A working knowledge of electricity is an asset in determining what conditions to use and in troubleshooting the electrophoretic separation, if necessary. For example, an unusually high or low voltage for a given current (milliampere) might indicate an improperly made buffer or an electrical leak in the chamber.

Safety Considerations

1. Never remove or insert high-voltage leads unless the power supply voltage is turned down to zero and the power supply is turned off. Always grasp high-voltage leads one at a time with one hand only. Never insert or remove high-voltage leads with both hands. This can shunt potentially lethal electricity through the chest and heart should electrical contact be made between a hand and a bare wire. On older or homemade instruments, the banana plugs may not be shielded and can still be connected to the

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power supply at the same time they make contact with a hand. Carefully inspect all cables and connections and replace frayed or exposed wires immediately.

2. Always start with the power supply turned off. Have the power supply controls turned all the way down to zero. Then hook up the gel apparatus: generally, connect the red high-voltage lead to the red outlet and the black high-voltage lead to the black outlet. Turn the power supply on with the controls set at zero and the high-voltage leads connected. Then, turn up the voltage, current, or power to the desired level. Reverse the process when the power supply is turned off: i.e., to disconnect the gel, turn the power supply down to zero, wait for the meters to read zero, turn off the power supply, and then disconnect the gel apparatus one lead at a time.

   CAUTION: *If the gel is first disconnected and then the power supply turned off, a considerable amount of electrical charge is stored internally. The charge will stay in the power supply over a long time. This will discharge through the outlets even though the power supply is turned off and can deliver an electrical shock.*

Ohm's Law and Electrophoresis

Understanding how a gel apparatus is connected to the power supply requires a basic understanding of Ohm's law: voltage = current × resistance, or $V = IR$. A gel can be viewed as a resistor and the power supply as the voltage and current source. Most power supplies deliver constant current or constant voltage. Some will also deliver constant power: power = voltage × current, or $VI = I^2R$. The discussion below focuses on constant current because this is the most common mode in vertical SDS-PAGE.

Most modern commercial equipment is color-coded so that the red or positive terminal of the power supply can simply be connected to the red lead of the gel apparatus, which goes to the lower buffer chamber. The black lead is connected to the black or negative terminal and goes to the upper buffer chamber. This configuration is designed to work with vertical slab gel electrophoreses in which negatively charged proteins or nucleic acids move to the positive electrode in the lower buffer chamber (an anionic system).

When a single gel is attached to a power supply, the negative charges flow from the negative cathode (black) terminal into the upper buffer chamber, through the gel, and into the lower buffer chamber. The lower buffer chamber is connected to the positive anode (red) terminal to complete the circuit. Thus, negatively charged molecules, such as SDS-coated proteins and nucleic acids, move from the negative cathode attached to the upper buffer chamber toward the positive anode attached to the lower chamber. SDS-PAGE is an anionic system because of the negatively charged SDS.

Occasionally, proteins are separated in cationic systems. In these gels, the proteins are positively charged because of the very low pH of the gel buffers (e.g., acetic acid/urea gels for histone separations) or the presence of a cationic detergent (e.g., cetyltrimethylammonium bromide, CTAB). Proteins move toward the negative electrode (cathode) in cationic gel systems, and the polarity is reversed compared to SDS-PAGE: the red lead from the lower buffer chamber is attached to the black outlet of the power supply, and the black lead from the upper buffer chamber is attached to the red outlet of the power supply.

Most SDS-PAGE separations are performed under constant current (consult instructions from the manufacturer to set the power supply for constant current operation). The resistance of the gel will increase during SDS-PAGE in the standard Laemmli system. If the current is constant, then the voltage will increase during the run as the resistance goes up.

Power supplies usually have more than one pair of outlets. The pairs are connected in parallel with one another internally. If more than one gel is connected directly to the outlets

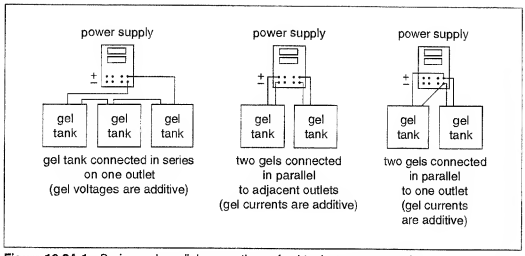

Figure 10.2A.1 Series and parallel connections of gel tanks to power supply.

of a power supply, then these gels are connected in parallel. In a parallel circuit, the voltage is the same across each gel. In other words, if the power supply reads 100 V, then each gel has 100 V across its electrodes. The total current, however, is the sum of the individual currents going through each gel. Therefore, under constant current it is necessary to increase the current for each additional gel that is connected to the power supply. Two identical gels require double the current to achieve the same starting voltages and electrophoresis separation times.

Multiple gel apparatuses can also be connected to one pair of outlets on a power supply. This is useful with older power supplies that have a limited number of outlets. When connecting several gel units to one outlet, make certain the connections between the units are shielded and protected from moisture. The gels can be connected in parallel or in series (Fig. 10.2A.1). In the case of two or more gels running off the same outlet in series, the current is the same for every gel. If 10 mA is displayed by the power supply meter, for example, each gel in series will experience 10 mA. The voltage, however, is additive for each gel. If one gel at a constant 10 mA produces 100 V, then two identical gels in series will produce 200 V (100 V each) and so on. Thus, the voltage can limit the number of units connected in series on low-voltage power supplies.

Gel thickness affects the above relationships. A 1.5-mm gel can be thought of as consisting of two 0.75-mm-thick gels run in parallel. Because currents are additive in parallel circuits, a 0.75-mm gel will require half the current of the 1.5-mm gel to achieve the same starting voltage and separation time. If a gel thickness is doubled, then the current must also be doubled. There are limits to the amount of current that can be applied. Thicker gels require more current, generating more heat that must be dissipated. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.

NOTE: Milli-Q-purified water or equivalent should be used throughout the protocols.

DENATURING (SDS) DISCONTINUOUS GEL ELECTROPHORESIS: LAEMMLI GEL METHOD

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT) is added during solubilization to reduce disulfide bonds.

This protocol is designed for a vertical slab gel with a maximum size of 0.75 mm × 14 cm × 14 cm. For thicker gels, or minigels (see Basic Protocol 2 and Support Protocol 3), the volumes of stacking and separating gels and the operating current must be adjusted. Additional protocols describe the preparation of ultrathin gels (see Alternate Protocol 4) and gradient gels (see Alternate Protocol 5), as well as the use of gel casters to make multiple gels, both single-concentration gels (see Support Protocol 1) and gradient gels (see Support Protocol 2).

Materials

Separating and stacking gel solutions (Table 10.2A.1)
H_2O-saturated isobutyl alcohol
1× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 10.2A.1)
Protein sample to be analyzed
2× and 1× SDS sample buffer (see recipe)
Protein molecular-weight-standards mixture (Table 10.2A.2)
6× SDS sample buffer (see recipe; optional)
1× SDS electrophoresis buffer (see recipe)

Electrophoresis apparatus: Protean II 16-cm cell (Bio-Rad) or SE 600/400 16-cm unit (Amersham Pharmacia Biotech) with clamps, glass plates, casting stand, and buffer chambers
0.75-mm spacers
0.45-μm filters (used in stock solution preparation)
25-ml Erlenmeyer side-arm flask
Vacuum pump with cold trap
0.75-mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
25- or 100-μl syringe with flat-tipped needle
Constant-current power supply (see Electricity and Electrophoresis, above)

Pour the separating gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates and two 0.75-mm spacers.

   If needed, clean the glass plates in liquid Alconox or RBS-35 (Pierce). These aqueous-based solutions are compatible with silver and Coomassie blue staining procedures.

2. Lock the sandwich to the casting stand.

3. Prepare the separating gel solution as directed in Table 10.2A.1, degassing using a rubber-stoppered 25-ml Erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specified amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.

   Table 10.2A.1 was prepared as a convenient summary to aid in the preparation of separating and stacking gels. The stacking gel is the same regardless of the separating gel used.

The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa (Table 10.2A.1).

4. Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates is ~11 cm.

   Use the solution immediately; otherwise it will polymerize in the flask.

   Sample volumes <10 µl do not require a stacking gel. In this case, cast the resolving gel as you normally would, but extend the resolving gel into the comb to form the well. The proteins are then separated under the same conditions as used when a stacking gel is present. Although this protocol works well with single-concentration gels, a gradient gel is recommended for maximum resolution (see Alternate Protocol 5).

5. Using another Pasteur pipet, slowly cover the top of the gel with a layer (~1 cm thick) of H_2O-saturated isobutyl alcohol, by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers.

   Be careful not to disturb the gel surface. The overlay provides a barrier to oxygen, which inhibits polymerization, and allows a flat interface to form during gel formation.

   The H_2O-saturated isobutyl alcohol is prepared by shaking isobutyl alcohol and H_2O in a separatory funnel. The aqueous (lower) phase is removed. This procedure is repeated several times. The final upper phase is H_2O-saturated isobutyl alcohol.

6. Allow the gel to polymerize 30 to 60 min at room temperature.

   A sharp optical discontinuity at the overlay/gel interface will be visible on polymerization. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED (N, N, N′, N′-tetramethylethylenediamine), or both. Ammonium persulfate solution should be made fresh before use. Ammonium persulfate should "crackle" when added to the water. If not, fresh ammonium persulfate should be purchased. Purchase TEMED in small bottles so, if necessary, a new previously unopened source can be tried.

Pour the stacking gel

7. Pour off the layer of H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Residual isobutyl alcohol can reduce resolution of the protein bands; therefore, it must be completely removed. The isobutyl alcohol overlay should not be left on the gel longer than 2 hr.

8. Prepare the stacking gel solution as directed in Table 10.2A.1.

   Use the solution immediately to keep it from polymerizing in the flask.

9. Using a Pasteur pipet, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~1 cm from the top of the plates.

   Be careful not to introduce air bubbles into the stacking gel.

10. Insert a 0.75-mm Teflon comb into the layer of stacking gel solution. If necessary, add additional stacking gel to fill the spaces in the comb completely.

    Again, be careful not to trap air bubbles in the tooth edges of the comb; they will cause small circular depressions in the well after polymerization that will lead to distortion in the protein bands during separation.

11. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

    A sharp optical discontinuity will be visible around wells on polymerization.

Table 10.2A.1 Recipes for Polyacrylamide Separating and Stacking Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in separating gel (%)[c]									
	5	6	7	7.5	8	9	10	12	13	15
30% acrylamide/ 0.8% bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4× Tris·Cl/SDS, pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution, 4×Tris·Cl/SDS, pH 8.8 (see reagents, below), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL (*3.9% acrylamide*)

In a 25-ml side-arm flask, mix 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4×Tris·Cl/SDS, pH 6.8 (see reagents, below), and 3.05 ml H_2O. Degas under vacuum 10 to 15 min. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED. Swirl gently to mix. Use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

REAGENTS USED IN GELS

30% acrylamide/0.8% bisacrylamide

Mix 30.0 g acrylamide and 0.8 g *N,N'*-methylenebisacrylamide with H_2O in a total volume of 100 ml. Filter the solution through a 0.45-µm filter and store at 4°C in the dark. The 2× crystallized grades of acrylamide and bisacrylamide are recommended. Discard after 30 days, as acrylamide gradually hydrolyzes to acrylic acid and ammonia.

CAUTION: *Acrylamide monomer is neurotoxic. A mask should be worn when weighing acrylamide powder. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.*

***4× Tris·Cl/SDS, pH 6.8** (0.5 M Tris·Cl containing 0.4% SDS)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter, add 0.4 g SDS, and store at 4°C up to 1 month.

***4× Tris·Cl/SDS, pH 8.8** (1.5 M Tris·Cl containing 0.4% SDS)*

Dissolve 91 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 2 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for a gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See annotation to step 3, Basic Protocol 1.

[d]Best to prepare fresh.

Prepare the sample and load the gel

12. Dilute a portion of the protein sample to be analyzed 1:1 (v/v) with 2× SDS sample buffer and heat 3 to 5 min at 100°C in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, dissolve the protein in 50 to 100 µl of 1× SDS sample buffer and boil 3 to 5 min at 100°C. Dissolve protein-molecular-weight standards mixture in 1× SDS sample buffer according to supplier's instructions; use these standards as a control (Table 10.2A.2).

For dilute protein solutions, consider adding 5:1 protein solution/6× SDS sample buffer to increase the amount of protein loaded. Proteins can also be concentrated by precipitation in acetone, ethanol, or trichloroacetic acid (TCA), but losses will occur.

For a 0.8-cm-wide well, 25 to 50 µg total protein in <20 µl is recommended for a complex mixture when staining with Coomassie blue, and 1 to 10 µg total protein is needed for samples containing one or a few proteins. If silver staining is used, 10- to 100-fold less protein can be applied (0.01 to 5 µg in <20 µl depending on sample complexity).

To achieve the highest resolution possible, the following precautions are recommended. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer (room temperature) directly to the 0°C sample (still on ice) in a screw-top microcentrifuge tube. Cap the tube to prevent evaporation, vortex, and transfer directly to a 100°C water bath for 3 to 5 min. Let immunoprecipitates dissolve for 1 hr at 56°C in 1× SDS sample buffer prior to boiling. DO NOT leave the sample in SDS sample buffer at room temperature without first heating to 100°C to inactivate proteases (see Critical Parameters and Troubleshooting). Endogenous proteases are very active in SDS sample buffer and will cause severe degradation of the sample proteins after even a few minutes at room temperature. To test for possible proteases, mix the sample with SDS sample buffer without heating and leave at room temperature for 1 to 3 hr. A loss of high-molecular-weight bands

Table 10.2A.2 Molecular Weights of Protein Standards for Polyacrylamide Gel Electrophoresis[a]

Protein	Molecular weight
Cytochrome *c*	11,700
α-Lactalbumin	14,200
Lysozyme (hen egg white)	14,300
Myoglobin (sperm whale)	16,800
β-Lactoglobulin	18,400
Trypsin inhibitor (soybean)	20,100
Trypsinogen, PMSF treated	24,000
Carbonic anhydrase (bovine erythrocytes)	29,000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36,000
Lactate dehydrogenase (porcine heart)	36,000
Aldolase	40,000
Ovalbumin	45,000
Catalase	57,000
Bovine serum albumin	66,000
Phosphorylase *b* (rabbit muscle)	97,400
β-Galactosidase	116,000
RNA polymerase, *E. coli*	160,000
Myosin, heavy chain (rabbit muscle)	205,000

[a]Protein standards are commercially available in kits (e.g., Amersham Pharmacia Biotech, Life Technologies, Bio-Rad, or Sigma).

and a general smearing of the banding pattern indicate a protease problem. Once heated, the samples can sit at room temperature for the time it takes to load samples.

13. Carefully remove the Teflon comb without tearing the edges of the polyacrylamide wells. After the comb is removed, rinse wells with 1× SDS electrophoresis buffer.

    The rinse removes unpolymerized monomer; otherwise, the monomer will continue to polymerize after the comb is removed, creating uneven wells that will interfere with sample loading and subsequent separation.

14. Using a Pasteur pipet, fill the wells with 1× SDS electrophoresis buffer.

    If well walls are not upright, they can be manipulated with a flat-tipped needle attached to a syringe.

15. Attach gel sandwich to upper buffer chamber following manufacturer's instructions.

16. Fill lower buffer chamber with the recommended amount of 1× SDS electrophoresis buffer.

17. Place sandwich attached to upper buffer chamber into lower buffer chamber.

18. Partially fill the upper buffer chamber with 1× SDS electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.

    Monitor the upper buffer chamber for leaks and if necessary, reassemble the unit. A slow leak in the upper buffer chamber may cause arcing around the upper electrode and damage the upper buffer chamber.

19. Using a 25- or 100-µl syringe with a flat-tipped needle, load the protein sample(s) into one or more wells by carefully applying the sample as a thin layer at the bottom of the wells. Load control wells with molecular weight standards. Add an equal volume of 1× SDS sample buffer to any empty wells to prevent spreading of adjoining lanes.

    Preparing the samples at approximately the same concentration and loading an equal volume to each well will ensure that all lanes are the same width and that the proteins run evenly. If unequal volumes of sample buffer are added to wells, the lane with the larger volume will spread during electrophoresis and constrict the adjacent lanes, causing distortions.

    The samples will layer on the bottom of the wells because the glycerol added to the sample buffer gives the solution a greater density than the electrophoresis buffer. The bromphenol blue in the sample buffer makes sample application easy to follow visually.

20. Fill the remainder of the upper buffer chamber with additional 1× SDS electrophoresis buffer so that the upper platinum electrode is completely covered. Do this slowly so that samples are not swept into adjacent wells.

Run the gel

21. Connect the power supply to the cell and run at 10 mA of constant current for a slab gel 0.75 mm thick, until the bromphenol blue tracking dye enters the separating gel. Then increase the current to 15 mA.

    For a standard 16-cm gel sandwich, 4 mA per 0.75-mm-thick gel will run ~15 hr (i.e., overnight); 15 mA per 0.75-mm gel will take 4 to 5 hr. To run two gels or a 1.5-mm-thick gel, simply double the current. When running a 1.5-mm gel at 30 mA, the temperature must be controlled (10° to 20°C) with a circulating constant-temperature water bath to prevent "smiling" (curvature in the migratory band). Temperatures <5°C should not be used because SDS in the running buffer will precipitate. If the level of buffer in the upper chamber decreases, a leak has occurred.

22. After the bromphenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Refer to Safety Considerations under Electricity and Electrophoresis.

Disassemble the gel

23. Discard electrode buffer and remove the upper buffer chamber with the attached gel sandwich.

24. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels.

25. Carefully slide one of the spacers halfway from the edge of the sandwich along its entire length. Use the exposed spacer as a lever to pry open the glass plate, exposing the gel.

26. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost during staining and drying. Proceed with protein detection.

The gel can be stained with Coomassie blue or silver (UNIT 10.6), or proteins can be electroeluted, electroblotted onto a polyvinylidene difluoride (PVDF) membrane for subsequent staining or sequence analysis, or transferred to a membrane for immunoblotting UNIT 10.8. If the proteins are radiolabeled, they can be detected by autoradiography (APPENDIX 3A).

ELECTROPHORESIS IN TRIS-TRICINE BUFFER SYSTEMS

ALTERNATE PROTOCOL 1

Separation of peptides and proteins under 10 to 15 kDa is not possible in the traditional Laemmli discontinuous gel system (see Basic Protocol 1). This is due to the comigration of SDS and smaller proteins, obscuring the resolution. Two approaches to obtain the separation of small proteins and peptides in the range of 5 to 20 kDa are presented: the following Tris-tricine method and a system using increased buffer concentrations (see Alternate Protocol 2). The Tris-tricine system uses a modified buffer to separate the SDS and peptides, thus improving resolution. Several precast gels are available for use with the tricine formulations (Table 10.2A.3).

Table 10.2A.3 Vertical Format Precast Gel Compatibility

Gel type and compatibility	Gel supplier			
	Bio-Rad	ISS/Daiichi	Jule	Novex
SDS-PAGE gel type offered				
Peptide (tricine)	×	×	×	×
Single concentration	×	×	×	×
Gradient	×	×	×	×
Minigel size	×	×	×	×
Standard gel size		×	×	
Compatibility of gel with equipment manufactured by				
Amersham Pharmacia Biotech		×	×	×
Bio-Rad	×	×	×	
Life Technologies	×	×	×	×
Novex		×		×
ISS/Daiichi		×	×	

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 10.2A.4)
2× tricine sample buffer (see recipe)
Peptide molecular-weight-standards mixture (Table 10.2A.5)
Cathode buffer (see recipe)
Anode buffer (see recipe)
Coomassie blue G-250 staining solution (see recipe)
10% (v/v) acetic acid

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11), using Table 10.2A.4 in place of Table 10.2A.1.

2. Prepare the sample (see Basic Protocol 1, step 12), but make the following changes for tricine gels. Substitute 2× tricine sample buffer for the 2× SDS sample buffer. Dilute an aliquot of the protein or peptide sample to be analyzed 1:1 (v/v) with 2× tricine sample buffer. Treat the sample at 40°C for 30 to 60 min prior to loading.

   If proteolytic activity is a problem, heating samples to 100°C for 3 to 5 min before loading the wells may be required (see Basic Protocol 1, annotation to step 12). Use the peptide molecular-weight-standards mixture for peptide separations (Table 10.2A.5).

3. Load the gel and set up the electrophoresis apparatus (see Basic Protocol 1, steps 13 to 20) with the following alterations. Remove comb and, using the tricine-containing

Table 10.2A.4 Recipes for Tricine Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% acrylamide/0.8% bisacrylamide	9.80 ml	1.62 ml
Tris·Cl/SDS, pH 8.45	10.00 ml	3.10 ml
H_2O	7.03 ml	7.78 ml
Glycerol	4.00 g (3.17 ml)	—
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately.

in a 50-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (Table 10.2A.1), Tris·Cl/SDS, pH 8.45 (see reagents, below), and H_2O. Add glycerol to separating gel only. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix, use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

ADDITIONAL REAGENTS USED IN GELS

Tris·Cl/SDS, pH 8.45 (3.0 M Tris·Cl containing 0.3% SDS)

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.45 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 1.5 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 30 ml of separating gel and 12.5 ml of stacking gel, which are adequate for two gels of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the Tris-tricine buffer system of Schagger and von Jagow (1987).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Best to prepare fresh.

Table 10.2A.5 Molecular Weights of Peptide Standards for Polyacrylamide Gel Electrophoresis[a]

Peptide	Molecular weight (Da)
Myoglobin (polypeptide backbone)	16,950
Myoglobin 1-131	14,440
Myoglobin 56-153	10,600
Myoglobin 56-131	8,160
Myoglobin 1-55	6,210
Glucagon	3,480
Myoglobin 132-153	2,510

[a]Peptide standards are commercially available from Sigma.

cathode buffer, or water, rinse once and fill wells. Fill the lower buffer chamber with anode buffer, assemble the unit, and attach the upper buffer chamber. Fill the upper buffer chamber with cathode buffer and load the samples.

4. Connect the power supply to the cell and run 1 hr at 30 V (constant voltage) followed by 4 to 5 hr at 150 V (constant voltage). Use heat exchanger to keep the electrophoresis chamber at room temperature.

5. After the tracking dye has reached the bottom of the separating gel, disconnect the power supply.

   Refer to Safety Considerations under Electricity and Electrophoresis.

   Coomassie blue G-250 is used as a tracking dye instead of bromphenol blue because it moves ahead of the smallest peptides.

6. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Stain proteins in the gel for 1 to 2 hr in Coomassie blue G-250 staining solution. Follow by destaining with 10% acetic acid, changing the solution every 30 min until background is clear (3 to 5 changes). For higher sensitivity, use silver staining as a recommended alternative.

   Prolonged staining and destaining will result in the loss of resolution of the smaller proteins (<10 kDa). Proteins diffuse within the gel and out of the gel, resulting in a loss of staining intensity and resolution.

NONUREA PEPTIDE SEPARATIONS WITH TRIS BUFFERS

ALTERNATE PROTOCOL 2

A simple modification of the traditional Laemmli buffer system presented in Basic Protocol 1, in which the increased concentration of buffers provides better separation between the stacked peptides and the SDS micelles, permits reasonable separation of peptides as small as 5 kDa.

***Additional Materials** (also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 10.2A.6)
2× SDS electrophoresis buffer (see recipe)
2× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 10.2A.1)

1. Prepare and pour the separating gel (see Basic Protocol 1, steps 1 to 6) using Table 10.2A.6 in place of Table 10.2A.1.

2. Prepare and pour the stacking gel (see Basic Protocol 1, steps 7 to 11), using 2× Tris·Cl/SDS, pH 8.8, rather than 1× Tris·Cl/SDS buffer, for rinsing the gel after removing the isobutyl alcohol overlay.

3. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20) and substitute 2× SDS electrophoresis buffer for the 1× SDS electrophoresis buffer.

Table 10.2A.5 lists the standards for small protein separations.

4. Run the gel (see Basic Protocol 1, steps 21 and 22).

Note that the separations will take ~25% longer than those using Basic Protocol 1. The increased buffer concentrations lead to faster transit through the stacking gel but lower mobility in the resolving gel.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

Proteins in the gel may now be stained.

Table 10.2A.6 Recipes for Modified Laemmli Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% acrylamide/0.8% bisacrylamide	10.00 ml	0.65 ml
8× Tris·Cl, pH 8.8	3.75 ml	—
4× Tris·Cl, pH 6.8	—	1.25 ml
10% (w/v) SDS	0.15 ml	50 µl
H_2O	1.00 ml	3.00 ml
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 8× Tris·Cl, pH 8.8 (separating gel) or 4× Tris·Cl, pH 6.8 (stacking gel), 10% SDS (see reagents, below), and H_2O. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

ADDITIONAL REAGENTS USED IN GELS

***4× Tris·Cl, pH 6.8** (0.5 M Tris·Cl)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

***8× Tris·Cl, pH 8.8** (3.0 M Tris·Cl)*

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

10% (w/v) SDS

Mix 1 g SDS in 10 ml of H_2O. Use immediately.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the modified Laemmli peptide separation system of Okajima et al. (1993).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

CONTINUOUS SDS-PAGE

ALTERNATE PROTOCOL 3

With continuous SDS-PAGE, the same buffer is used for both the gel and electrode solutions. Although continuous gels lack the resolution of the discontinuous systems, they are extremely versatile, less prone to mobility artifacts, and much easier to prepare. The stacking gel is omitted.

***Additional Materials** (also see Basic Protocol 1)*

Separating gel solution (Table 10.2A.7)
2× and 1× phosphate/SDS sample buffer (see recipe)
1× phosphate/SDS electrophoresis buffer (see recipe)

1. Prepare and pour a single separating gel (see Basic Protocol 1, steps 1 to 4), except use solutions in Table 10.2A.7 in place of those in Table 10.2A.1 and fill the gel sandwich to the top. Omit the stacking gel. Insert the comb (see Basic Protocol 1, step 10) and allow the gel to polymerize 30 to 60 min at room temperature.
2. Mix the protein sample 1:1 with 2× phosphate/SDS sample buffer and heat to 100°C for 2 min.

   For large sample volumes or samples suspended in high ionic strength buffers (>50 mM), dialyze against 1× sample buffer prior to electrophoresis. Note that the precautions about proteases (see Basic Protocol 1, step 12) apply here.

Table 10.2A.7 Recipes for Separating Gels for Continuous SDS-PAGE[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in separating gel (%)[c]										
	5	6	7	8	9	10	11	12	13	14	15
30% acrylamide/ 0.8% bisacrylamide	2.5	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50
4× phosphate/SDS, pH 7.2	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 4× phosphate/SDS, pH 7.2, and H_2O. Degas under vacuum about 5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS

***4× phosphate/SDS, pH 7.2** (0.4 M sodium phosphate/0.4% SDS)*

Mix 46.8 g $NaH_2PO_4 \cdot H_2O$, 231.6 g $Na_2HPO_4 \cdot 7\ H_2O$, and 12 g SDS in 3 liters H_2O.

Store at 4°C for up to 3 months.

[a]The recipes produce 15 ml of separating gel, which is adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the original continuous phosphate buffer system of Weber et al. (1972). The stacking gel is omitted.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.

[d]Best to prepare fresh.

3. Assemble the electrophoresis apparatus and load the sample (see Basic Protocol 1, steps 13 to 20), using the phosphate/SDS electrophoresis buffer and loading empty wells with 1× phosphate/SDS sample buffer.

4. Connect the power supply and start the run with 15 mA per 0.75-mm-thick gel until the tracking dye has entered the gel. Continue electrophoresis at 30 mA for 3 hr (5% gel), 5 hr (10% gel), 8 hr (15% gel), or until the dye reaches the bottom of the gel.

   Use temperature control if available to maintain the gel at 15° to 20°C. SDS will precipitate below 15°C in this system.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

   See Safety Considerations in introduction. Proteins in the gel may now be stained.

ALTERNATE PROTOCOL 4

CASTING AND RUNNING ULTRATHIN GELS

Ultrathin gels provide superb resolution but are difficult to handle. In this application, gels are cast on Gel Bond, a Mylar support material. Silver staining is recommended for the best resolution. Combs and spacers for gels <0.5 mm thick are not readily available for most protein electrophoresis units. However, by adapting combs and spacers used for DNA sequencing, casting gels from 0.2 to 0.5 mm thick is straightforward.

***Additional Materials** (also see Basic Protocol 1)*

95% (v/v) ethanol
Gel Bond (FMC BioProducts) cut to a size slightly smaller than the gel plate dimensions
Glue stick
Ink roller (available from art supply stores)
Combs and spacers (0.19 to 0.5 mm; sequencing gel spacers and combs can be cut to fit)

1. Wash gel plates with water-based laboratory detergent followed by successive rinses with hot tap water, deionized water, and finally 95% ethanol. Allow to air dry.

   Gel plates must be extremely clean for casting thin gels.

   Gloves are needed throughout these procedures to prevent contamination by proteins on the surface of skin.

2. Apply a streak of adhesive from a glue stick to the bottom edge of the glass plate. Quickly position the Gel Bond with the hydrophobic side down (a drop of water will bead up on the hydrophobic surface). Apply pressure with Kimwipe tissue to attach the Gel Bond firmly to the plate. Finally, pull the top portion of the Gel Bond back, place a few drops of water underneath, and roll flat with an ink roller.

   Make sure the Gel Bond does not extend beyond the edges of the upper and lower sealing surface of the plate. This will cause it to buckle on sealing. Reposition the Gel Bond if needed to prevent it from extending beyond the glass plate. Material may also be trimmed to fit flush with the plate edge.

3. Assemble the gel cassette according to the manufacturer's instructions (also see Basic Protocol 1, steps 1 and 2). Just prior to assembly, blow air over the surface of both the Gel Bond and the opposing glass surface to remove any particulate material (e.g., dust).

   Sequencing gel spacers can be easily adapted. First, cut the spacers slightly longer than the length of the gel plate. Position a spacer along each edge of the glass plate and assemble the gel sandwich, clamping in place. With a razor blade, trim the excess spacer at top and bottom to get a reusable spacer exactly the size of the plate.

4. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 3 to 9). In place of the Teflon comb, insert a square well sequencing comb cut to fit within the gel sandwich. Allow the stacking gel to polymerize 30 to 45 min at room temperature.

   Less solution is needed for ultrathin gels. For example, a 0.5-mm-thick gel requires 33% less gel solution than a 0.75-mm gel.

5. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20).

   When preparing protein samples for ultrathin gels, 3 to 4 µl at 5 µg protein/µl is required for Coomassie blue R-250 staining, whereas 10-fold less is needed for silver staining.

6. Run the gel (see Basic Protocol 1, steps 21 and 22), except conduct the electrophoresis at 7 mA/gel (0.25-mm-thick gels) or 14 mA/gel (0.5-mm-thick gels) for 4 to 5 hr.

7. When the separation is complete, disassemble the unit and remove the gel (see Basic Protocol 1, steps 23 to 26) with the Gel Bond still attached. With a gloved hand, wash away the adhesive material from the back of the Gel Bond under a stream of water before proceeding to protein detection.

   Either Coomassie blue or silver staining may be used, but silver staining produces particularly fine resolution with thin Gel Bond–backed gels. Compared to staining thicker (>0.75 mm) gels, thin (<0.75 mm) gels stain and destain more quickly. Although the optimum staining times must be empirically determined, all steps in Coomassie blue and silver staining procedures are generally reduced by half.

CASTING MULTIPLE SINGLE-CONCENTRATION GELS

SUPPORT PROTOCOL 1

Casting multiple gels at one time has several advantages. All the gels are identical, so sample separation is not affected by gel-to-gel variation. Furthermore, casting ten gels is only slightly more difficult than casting two gels. Once cast, gels can be stored for several days in a refrigerator.

***Additional Materials** (also see Basic Protocol 1)*

Separating and stacking gels for single-concentration gels (Table 10.2A.8)
H_2O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Amersham Pharmacia Biotech)
100-ml disposable syringe and flat-tipped needle
Extra plates and spacers
14 × 14–cm acrylic blocks or polycarbonate sheets
250- and 500-ml side-arm flasks (used in gel preparation)
Long razor blade *or* plastic wedge (Wonder Wedge, Amersham Pharmacia Biotech)
Resealable plastic bags

Pour the separating gel

1. Assemble the multiple gel caster according to the manufacturer's instructions.

   With the Amersham Pharmacia Biotech unit make sure to insert the large triangular space filler plug in the base of the caster. The plug is removed when casting gradient gels (see Support Protocol 2).

2. Assemble glass sandwiches and stack them in the casting chamber. Stack up to ten 1.5-mm gels and fill in extra space with acrylic blocks or polycarbonate sheets to hold the sandwiches tightly in place. Make sure the spacers are straight along the top, right, and left edges of the glass plates and that all edges of the stack are flush.

   The presence of loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis. Polycarbonate inhibits gel polymerization.

Table 10.2A.8 Recipes for Multiple Single-Concentration Polyacrylamide Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in separating gel (%)[c]										
	5	6	7	8	9	10	11	12	13	14	15
30% acrylamide/0.8% bisacrylamide	52	62	72	83	93	103	114	124	134	145	155
4× Tris·Cl/SDS, pH 8.8	78	78	78	78	78	78	78	78	78	78	78
H_2O	181	171	160	150	140	129	119	109	98	88	78
10% (w/v) ammonium persulfate[d]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TEMED	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21

Preparation of separating gel

In a 500-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 4× Tris·Cl/SDS, pH 8.8 (Table 10.2A.1), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately.

STACKING GEL

In a 250-ml side-arm flask, mix 13.0 ml 30% acrylamide/0.8% bisacrylamide solution, 25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 10.2A.1), and 61 ml H_2O. Degas under vacuum ~5 min. Add 0.25 ml 10% ammonium persulfate and 50 µl TEMED. Swirl gently to mix. Use immediately. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

[a]The recipes produce about 300 ml of separating gel and 100 ml of stacking gel, which are adequate for ten gels of dimensions 1.5 mm × 14 cm × 14 cm. Volumes were measured using 1.5-mm spacers. For thinner spacers or fewer gels, calculate volumes using the equation in the annotation to step 4. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.

[d]Best to prepare fresh.

Therefore, if polycarbonate sheets are placed in the caster before and after the set of glass sandwiches, the entire set will slide out as one block after polymerization. Placing polycarbonate sheets between each gel sandwich makes them easier to separate from one another after polymerization.

3. Place the front sealing plate on the casting chamber, making sure the stack fits snugly. Secure the plate with four spring clamps and tighten the bottom thumb screws.

4. Prepare the separating (resolving) gel solution (Table 10.2A.8).

   A 12-cm separating gel with a 4-cm stacking gel is recommended.

   If fewer than ten gels are prepared (Table 10.2A.8), use the following formula to estimate the amount of separating gel volume needed:

   Volume = gel number × height (cm) × width (cm) × thickness (cm) + 4 × gel number + 10 ml.

5. Using a 100-ml disposable syringe with flat-tipped needle, inject the resolving gel solution down the side of one spacer into the multiple caster. A channel in the silicone plug distributes the solution throughout the whole caster. Avoid introducing bubbles by giving the caster a quick tap on the benchtop once the caster is filled.

6. Overlay the center of each gel with 100 µl H_2O-saturated isobutyl alcohol and let polymerize for 1 to 2 hr.

7. Drain off the overlay and rinse the surface with 1× Tris·Cl/SDS, pH 8.8. If the gels will not be used immediately, skip to step 12.

Pour the stacking gel

8. Prepare the stacking gel solution either singly (see Basic Protocol 1, step 8) or for all the gels at once (Table 10.2A.8).

   The stacking gel solution should be prepared just before pouring the gel.

9. Fill each sandwich in the caster with stacking gel solution.

10. Insert a comb into each sandwich and let the gel polymerize for 2 hr.

    Insert the combs at a 45° angle to avoid trapping air underneath the comb teeth. Air bubbles will inhibit polymerization and cause dents in the wells and a distorted pattern of protein bands.

11. Remove the combs and rinse wells with 1× SDS electrophoresis buffer.

Remove the gels from the caster

12. Remove the gels from the caster and separate by carefully inserting a long razor blade or knife between each gel sandwich.

    A plastic wedge (Amersham Pharmacia Biotech's Wonder Wedge) also works well.

13. Clean the outside of each gel plate with running water to remove the residual polymerized and unpolymerized acrylamide.

14. Overlay gels to be stored with 1× Tris·Cl/SDS, pH 8.8, place in a resealable plastic bag, and store at 4°C until needed (up to 1 week).

SEPARATION OF PROTEINS ON GRADIENT GELS

ALTERNATE PROTOCOL 5

Gels that consist of a gradient of increasing polyacrylamide concentration resolve a much wider size range of proteins than standard uniform-concentration gels (see Critical Parameters and Troubleshooting). The protein bands, particularly in the low-molecular-weight range, are also much sharper. Unlike single-concentration gels, gradient gels separate proteins in a way that can be represented easily to give a linear plot from 10 to 200 kDa. This facilitates molecular weight estimations.

The quantities given below provide separating gel solution sufficient for two 0.75-mm gels (~7 ml of each concentration) or one 1.5-mm gel (~14 ml of each concentration). Volumes can be adjusted to accommodate gel sandwiches of different dimensions.

Additional Materials *(also see Basic Protocol 1)*

Light and heavy acrylamide gel solutions (Table 10.2A.9 and Table 10.2A.10)
Bromphenol blue (optional; for checking practice gradient)
TEMED

Gradient maker (30 to 50 ml, Amersham Pharmacia Biotech SG30 or SG50; *or* 30 to 100 ml, Bio-Rad 385)
Tygon tubing with micropipet tip
Peristaltic pump (optional; e.g., Markson A-13002, A-34040, or A-34105 minipump)
Whatman 3MM filter paper

Set up the gradient maker and prepare the gel solutions

1. Assemble the magnetic stirrer and gradient maker on a ring stand as shown in Figure 10.2A.2. Connect the outlet valve of the gradient maker to Tygon tubing attached to a micropipet tip that is placed over the vertical gel sandwich. If desired, place a peristaltic pump in line between the gradient maker and the gel sandwich.

   A peristaltic pump will simplify casting by providing a smooth flow rate.

2. Place a small stir-bar into the mixing chamber of the gradient maker (i.e., the chamber connected to the outlet).

3. Using the recipes in Table 10.2A.9 and Table 10.2A.10, prepare light and heavy acrylamide gel solutions. Do not add ammonium persulfate until just before use (step 7).

4. With the outlet port and interconnecting valve between the two chambers closed, pipet 7 ml of light (low-concentration) acrylamide gel solution into the reservoir chamber for one 0.75-mm-thick gradient gel.

   Recommended gradient ranges are 5% to 20% for most applications (to separate proteins of 10 to several hundred kilodaltons).

   A practice run with heavy and light solutions is recommended. Bromphenol blue should be added to the heavy solution to demonstrate linearity of the practice gradient.

5. Open the interconnecting valve briefly to allow a small amount (~200 µl) of light solution to flow through the valve and into the mixing chamber.

   The presence of air bubbles in the interconnecting valve may obstruct the flow between chambers during casting.

   Deaeration is not recommended for either the light or heavy solution. Omitting the deaeration will allow polymerization to proceed more slowly, letting the gradient establish itself in the gel sandwich before polymerization takes place.

6. Add 7 ml of heavy (high-concentration) acrylamide gel solution to the mixing chamber.

   Keep the heavy solution on ice until use. Once the ammonium persulfate is added to the heavy solution, it will polymerize without TEMED, albeit more slowly; keeping the solution on ice prevents this. The gel solution will come to room temperature during casting. The higher the percentage of acrylamide, the more severe the problem of premature polymerization.

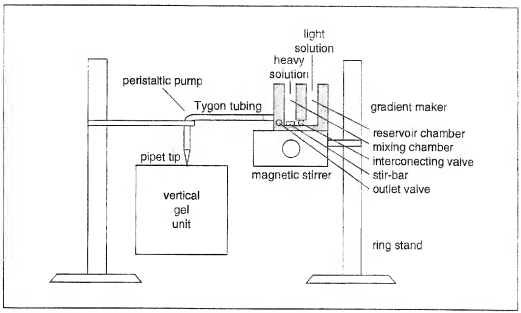

Figure 10.2A.2 Gradient gel setup. A peristaltic pump, though not required, will provide better control.

Table 10.2A.9 Light Acrylamide Gel Solutions for Gradient Gels[a]

Stock solution	Acrylamide concentration of light gel solution (%)[b]									
	5	6	7	8	9	10	11	12	13	14
30% acrylamide/ 0.8% bisacrylamide[c]	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

[a]To survey proteins ≥10 kDa, 5%-20% gradient gels are recommended. To expand the range between 10 and 200 kD, a 10%-20% gel is recommended.

[b]Numbers in body of table are milliliters of stock solution. Deaeration is not required. Keep solution at room temperature prior to adding TEMED no longer than 1 hr.

[c]See Table 10.2A.1 for preparation.

[d]Best to prepare fresh.

Table 10.2A.10 Heavy Acrylamide Gel Solutions for Gradient Gels[a]

Stock solution	Acrylamide concentration of heavy gel solution (%)[b]										
	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/ 0.8% bisacrylamide[c]	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0
Sucrose (g)	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
10% (w/v) ammonium persulfate[b,d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

[a]Deaeration is not recommended for gradient gels.

[b]Numbers in body of table are milliliters of stock solution (except sucrose). Do not add the ammonium persulfate until just before use. The heavy acrylamide will polymerize, albeit more slowly, without the addition of TEMED. Keep the heavy solution on ice after adding ammonium persulfate.

[c]See Table 10.2A.1 for preparation.

[d]Best to prepare fresh.

7. Add the specified amount of 10% ammonium persulfate and ~2.3 µl TEMED per 7 ml acrylamide solution to each chamber. Mix the solutions in each chamber with a disposable pipet.

Form the gradient and cast the gel

8. Open the interconnecting valve completely.

   Some of the heavy solution will flow back into the reservoir chamber containing light solution as the two chambers equilibrate. This will not affect the formation of the gradient.

9. Turn on the magnetic stirrer and adjust the rate to produce a slight vortex in the mixing chamber.

10. Open the outlet of the gradient maker slowly. Adjust the outlet valve to a flow rate of 2 ml/min. If using a peristaltic pump, calibrate the flow rate with a graduated cylinder prior to casting the gel.

    Some adjustment of the flow rate may be necessary during casting. If the light solution is not flowing into the mixing chamber, a bubble may be caught in the interconnecting valve.

Quickly close the outlet and cover the top of the reservoir chamber with a gloved thumb. Push down with the thumb to increase the pressure in the chamber and force the air bubble out of the center valve.

11. Fill the gel sandwich from the top. Place the pipet tip against one side of the sandwich so the solution flows down one plate only. The heavy solution will flow into the sandwich first, followed by progressively lighter solution.

12. Watch as the last of the light solution drains into the outlet tube and adjust the flow rate to ensure that the last few milliliters of solution do not flow quickly into the gel sandwich and disturb the gradient.

13. Overlay the gradient gel with H_2O-saturated isobutyl alcohol. Allow the gel to polymerize ~1 hr.

    In this gel system, the gel will polymerize from the bottom (i.e., heavy solution) up. Because polymerization is an exothermic reaction, heat can be felt evolving from the bottom of the gel sandwich during polymerization. A sharp optical discontinuity at the gel-overlay interface indicates that polymerization has occurred. In general, 1 hr is adequate for polymerization.

14. Remove the H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8. Cast the stacking gel (see Basic Protocol 1, steps 8 to 11).

    The gel can be covered with 1× Tris·Cl/SDS, pH 8.8, sealed in a plastic bag, and stored for up to 1 week.

Load and run the gel

15. Prepare the protein sample and protein molecular-weight-standards mixture. Load and run the gel (see Basic Protocol 1, steps 13 to 26).

    The gel can be stained with Coomassie blue or silver (UNIT 10.6).

16. After staining, dry the gels onto Whatman 3MM or equivalent filter paper.

    Gradient gels >0.75 mm thick require special handling during drying to prevent cracking. The simplest approach to drying gradient gels is to use thin gels: ≤0.75-mm gradient gels with ≤20% acrylamide solutions will dry without cracking as long as the vacuum pump is working properly and the cold trap is dry at the onset of drying. For gradient gels >0.75 mm thick, add 3% (w/v) glycerol to the final destaining solution to help prevent cracking. Another method is to dehydrate and shrink the gel in 30% methanol for up to 3 hr prior to drying. Then place the gel in distilled water for 5 min before drying.

SUPPORT PROTOCOL 2

CASTING MULTIPLE GRADIENT GELS

Casting gradient gels in a multiple gel caster has several advantages. In addition to the time savings, batch casting gives gels that are essentially identical. This is particularly important for gradient gels, where slight variations in casting technique can cause variations in protein mobility. The gels may be stored for up to 1 week after casting to ensure internal consistency from run to run during the week. Furthermore, gels with several ranges of concentrations (e.g., 5% to 20% and 10% to 20% acrylamide) can be cast and stored, giving much more flexibility to optimize separations.

Additional Materials *(also see Alternate Protocol 5)*

Plug solution (see recipe)
Light and heavy acrylamide gel solutions for multiple gradient gels (Table 10.2A.11 and Table 10.2A.12)
TEMED
H_2O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Amersham Pharmacia Biotech)
Peristaltic pump (25 ml/min)
500- or 1000-ml gradient maker (Bio-Rad, Amersham Pharmacia Biotech)
Tygon tubing

Set up system and pour separating gel

1. Assemble the multiple caster as in casting multiple single-concentration gels (see Support Protocol 1, steps 1 to 3), making sure to remove the triangular space filler plugs in the bottom of the caster.

   The plug is used only when casting single-concentration gels.

2. Set up the peristaltic pump (Fig. 10.2A.3). Using a graduated cylinder and water, adjust the flow rate so that the volume of the gradient solution plus volume of plug solution is poured in ~15 to 18 min (~25 ml/min).

3. Set up the gradient maker. Close all valves and place a stir-bar in the mixing chamber, which is the one with the outlet port. Attach one end of a piece of Tygon tubing to the outlet of the gradient maker. Run the other end of the tubing through the peristaltic pump and attach it to the red inlet port at the bottom of the caster.

   Choose a gradient maker that holds no more than four times the total volume of the gradient solution to be poured (i.e., a 1000- or 500-ml gradient maker).

4. Prepare solutions for the gradient maker (Table 10.2A.11 and Table 10.2A.12).

5. Add the TEMED to both heavy and light solutions (54 µl/165 ml) and immediately pour the light (low-concentration) solution into the mixing chamber (the one with the port). Open the mixing valve slightly to allow the tunnel to fill and to avoid air bubbles. Close the valve again and pour the heavy (high-concentration) acrylamide solution into the reservoir chamber.

6. Start the magnetic stirrer and open the outlet valve; then start the pump and open the mixing valve.

   In units for casting multiple gels, acrylamide solution flows in from the bottom. To use a multiple casting unit, the light solution is placed in the mixing chamber and the heavy solution in the reservoir. This is the reverse of casting a single gel (see Alternate Protocol 5).

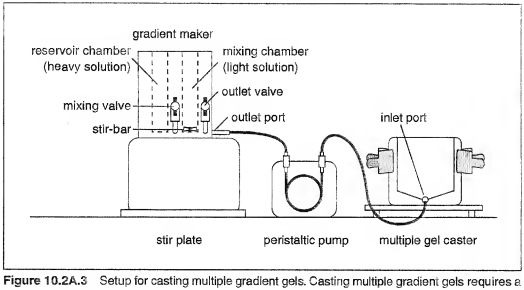

Figure 10.2A.3 Setup for casting multiple gradient gels. Casting multiple gradient gels requires a peristaltic pump and a multiple gel caster. Gel solution is introduced through the bottom of the multiple caster.

Table 10.2A.11 Light Acrylamide Gel Solutions for Multiple Gradient Gels[a,b]

Stock solution	Acrylamide concentration of light separating gel solution (%)[c]									
	5	6	7	8	9	10	11	12	13	14
30% acrylamide/ 0.8% bisacrylamide[d]	28	33	39	44	50	55	61	66	72	77
4× Tris·Cl/SDS, pH 8.8[d]	41	41	41	41	41	41	41	41	41	41
H_2O	96	91	85	80	74	69	63	58	52	47
10% (w/v) ammonium persulfate[e]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

[a]To survey proteins ≥10 kDa, 5%–20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10%–20% gel is recommended.

[b]Recipes produce ten 1.5-mm-thick gradient gels with 10 ml extra solution to account for losses in tubing.

[c]Numbers in body of table are milliliters of stock solution. Deaeration is not required. Keep solution at room temperature prior to adding TEMED no longer than 1 hr.

[d]See Table 10.2A.1 for preparation.

[e]Best to prepare fresh.

Table 10.2A.12 Heavy Acrylamide Gel Solutions for Multiple Gradient Gels[a,b]

Stock solution	Acrylamide concentration of heavy gel solution (%)[c]										
	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/ 0.8% bisacrylamide[d]	55	61	66	72	77	83	88	94	99	105	110
4× Tris·Cl/SDS, pH 8.8[d]	41	41	41	41	41	41	41	41	41	41	41
H_2O	55	50	44	39	33	28	22	17	11	5.5	0
Sucrose (g)	25	25	25	25	25	25	25	25	25	25	25
10% (w/v) ammonium persulfate[e]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

[a]Deaeration is not recommended for gradient gels.

[b]Recipes produce 10 ml extra solution to account for losses in tubing.

[c]Numbers in body of table are milliliters of stock solution (except sucrose). Do not add the ammonium persulfate until just before use. The heavy acrylamide will polymerize, albeit more slowly, without the addition of TEMED. Keep the heavy solution on ice after adding ammonium persulfate.

[d]See Table 10.2A.1 for preparation.

[e]Best to prepare fresh.

Thus, light solution enters the multiple caster first, followed by progressively heavier solution. Finally, the acrylamide solution is stabilized in the multiple caster with a heavy plug solution and allowed to polymerize (see step 8 and manufacturer's instructions).

7. When almost all the acrylamide solution is gone from the gradient maker, stop the pump and close the mixing valve. Tilt the gradient maker toward the outlet side and remove the last milliliters of the mix. Do not allow air bubbles to enter the tubing.

8. Add the plug solution to the mixing chamber and start the pump. Make sure that no bubbles are introduced. Continue pumping until the bottom of the caster is filled with plug solution to just below the glass plates; then turn off the pump. Clamp the tubing close to the red port of the casting chamber.

9. Quickly overlay each separate gel sandwich with 100 µl H_2O-saturated isobutyl alcohol. Use the same amount on each sandwich.

Failure to use the same amount of overlay solution will cause the gel sandwiches to polymerize at different heights.

10. Drain off the overlay and rinse the surface of the gels with 1× Tris·Cl/SDS, pH 8.8.

Pour stacking gel and remove gels from caster

11. Prepare and cast the stacking gel as in casting multiple single-concentration gels (see Support Protocol 1, steps 8 to 11).

12. Remove gels from the caster and clean the gel sandwiches (see Support Protocol 1, steps 12 and 13). Store gels, if necessary, according to the instructions for multiple single-concentration gels (see Support Protocol 1, step 14).

ELECTROPHORESIS IN SINGLE-CONCENTRATION MINIGELS

BASIC PROTOCOL 2

Separation of proteins in a small-gel format is becoming increasingly popular for applications that range from isolating material for peptide sequencing to performing routine protein separations. The unique combination of speed and high resolution is the foremost advantage of small gels. Additionally, small gels are easily adapted to single-concentration, gradient, and two-dimensional SDS-PAGE procedures. The minigel procedures described are adaptations of larger gel systems.

This protocol describes the use of a multiple gel caster. The caster is simple to use, and up to five gels can be prepared at one time with this procedure. Single gels can be prepared using adaptations in the manufacturer's instructions. A multiple gel caster is the only practical way to produce small linear polyacrylamide gradient gels (see Support Protocol 3).

Materials

Minigel vertical gel unit (Amersham Pharmacia Biotech Mighty Small SE 250/280 *or* Bio-Rad Mini-Protean II) with glass plates, clamps, and buffer chambers
0.75-mm spacers
Multiple gel caster (Amersham Pharmacia Biotech SE-275/295 *or* Bio-Rad Mini-Protean II multicasting chamber)
Acrylic plate (Amersham Pharmacia Biotech SE-217 *or* Bio-Rad 165-1957) or polycarbonate separation sheet (Amersham Pharmacia Biotech SE-213 *or* Bio-Rad 165-1958)
10- and 50-ml syringes
Combs (Teflon, Amersham Pharmacia Biotech SE-211A series or Bio-Rad Mini-Protean II)
Long razor blade
Micropipet

Additional reagents and equipment for standard denaturing SDS-PAGE (see Basic Protocol 1)

Pour the separating gel

1. Assemble each gel sandwich by stacking, in order, the notched (Amersham Pharmacia Biotech) or small rectangular (Bio-Rad) plate, 0.75-mm spacers, and the larger rectangular plate. Be sure to align the spacers properly, with the ends flush with the top and bottom edge of the two plates, when positioning the sandwiches in the multiple gel caster (Fig. 10.2A.4).

The protocol described is basically for the Amersham Pharmacia Biotech system. For other systems, make adjustments according to the manufacturer's instructions. Alternatively, precast minigels can be purchased from a number of suppliers (see Table 10.2A.3).

The multiple casters from Amersham Pharmacia Biotech have a notch in the base designed for casting gradient gels. A silicone rubber insert fills up this space when casting

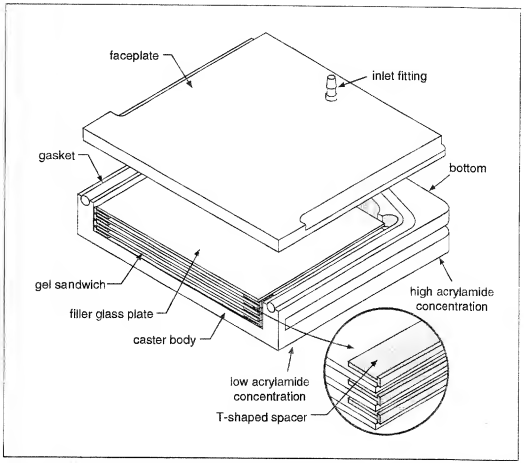

Figure 10.2A.4 Minigel sandwiches positioned in the multiple gel caster. Extra glass or acrylic plates or polycarbonate sheets are used to fill any free space in the caster and to ensure that the gel sandwiches are held firmly in place.

single-concentration gels. The Amersham Pharmacia Biotech spacers are T-shaped to prevent slipping. The flanged edge of the spacer must be positioned against the outside edge of the glass plate. Placing a sheet of wax paper between the gel sandwiches will help separate the sandwiches after polymerization.

2. Fit the gel sandwiches tightly in the multiple gel caster. Use an acrylic plate or polycarbonate separation sheet to eliminate any slack in the chamber.

   Loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis.

3. Place the front faceplate on the caster, clamp it in place against the silicone gasket, and verify alignment of the glass plates and spacers.

4. Prepare the separating gel solution as directed in Table 10.2A.1. For five 0.75-mm-thick gels, prepare ~30 ml solution (i.e., double the volumes listed).

   To compute the total gel volume needed, multiply the area of the gel (e.g., 7.3 × 8.3 cm) by the thickness of the gel (e.g., 0.75 mm) and then by the number of gels in the caster. If needed, add ~4 to 5 ml of extra gel solution to account for the space around the outside of the gel sandwiches.

   Do not add TEMED and ammonium persulfate until just before use.

5. Fill a 50-ml syringe with the separating gel solution and slowly inject it into the caster until the gels are 6 cm high, allowing 1.5 cm for the stacking gel.

6. Overlay each gel with 100 µl H_2O-saturated isobutyl alcohol. Allow the gels to polymerize for ~1 hr.

Pour the stacking gel

7. Remove the isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Stacking gels can be cast one at a time with the gel mounted on the electrophoresis unit, or all at once in the multiple caster.

8. Practice placing a comb in the gel sandwiches before preparing the stacking gel solution. Press the comb against the rectangular or taller plate so that all teeth of the comb are aligned with the opening in the gel sandwich, then insert into the sandwich. Remove combs after practicing.

9. Prepare the stacking gel solution (2 ml per gel) as directed in Table 10.2A.1. Fill a 10-ml syringe with stacking gel solution and inject the solution into each gel sandwich.

10. Insert combs, taking care not to trap bubbles. Allow gels to polymerize 1 hr.

11. Remove the front faceplate. Carefully pull the gels out of the caster, using a long razor blade to separate the sandwiches.

    If the gels are left to polymerize for prolonged periods, they will be difficult to remove from the caster.

    The gels can be stored tightly wrapped in plastic wrap with the combs left in place inside a sealable bag to prevent drying for ~1 week. Without the stacking gel, the separating gel can be stored for 2 to 3 weeks. Keep gels moist with 1× Tris·Cl/SDS, pH 8.8, at 4°C. Do not store gels in the multiple caster.

Prepare the sample, load the gel, and conduct electrophoresis

12. Remove the combs and rinse the sample wells with 1× SDS electrophoresis buffer. Place a line indicating the bottom of each well on the front glass plate with a marker.

13. Fill the upper and lower buffer chambers with 1× SDS electrophoresis buffer. The upper chamber should be filled to 1 to 2 cm over the notched plate.

14. Prepare the protein sample and protein-standards mixture (see Basic Protocol 1, step 12).

15. Load the sample using a micropipet. Insert the pipet tip through the upper buffer and into the well. The mark on the glass plate will act as a guide. Dispense the sample into the well.

    For a complex mixture, 20 to 25 µg protein in 10 µl SDS sample buffer will give a strongly stained Coomassie blue pattern. Much smaller amounts (1 to 5 µg) are required for highly purified proteins, and a 10- to 100-fold smaller amount of protein in the same volume (e.g., 10 µl) is required for silver staining.

16. Electrophorese samples at 10 to 15 mA per 0.75-mm gel until the dye front reaches the bottom of the gel (~1 to 1.5 hr).

17. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Proceed with detection of proteins.

PREPARING MULTIPLE GRADIENT MINIGELS

Polyacrylamide gradients not only enhance the resolution of larger-format gels but also greatly improve protein separation in the small format. Casting gradient minigels one at a time is not generally feasible because of the small volumes used, but multiple gel casters make it easy to cast several small gradient gels at one time. The gels are cast from the bottom in multiple casters, with the light acrylamide solution entering first. This is the opposite of casting one gel at a time, in which the heavy solution enters from the top of the gel sandwich and flows down to the bottom.

Additional Materials *(also see Basic Protocol 2)*

Plug solution (see recipe)

Additional reagents and equipment for preparing gradient gels (see Alternate Protocol 5)

Set up the system and prepare the gel solutions

1. Assemble minigel sandwiches in the multiple gel caster as described for single-concentration minigels (see Basic Protocol 2, steps 1 to 3).

2. Set up the 30-ml gradient maker, magnetic stirrer, peristaltic pump (optional), and Tygon tubing as in Figure 10.2A.3. Connect the outlet of the 30-ml gradient maker to the inlet at the base of the front faceplate of the caster.

   The monomer solution will be introduced through the inlet at the bottom of the front faceplate of the caster first, followed by progressively heavier solution.

3. Prepare light (Table 10.2A.9) and heavy (Table 10.2A.10) acrylamide gel solutions. Use ~12 ml of each solution for five 0.75-mm-thick minigels.

   Adjust volumes if a different thickness or number of gels is needed. Do not add ammonium persulfate until just before use. Deaeration is not recommended for gradient gels.

4. With the outlet and interconnecting valve closed, add the heavy solution to the reservoir chamber. Briefly open the interconnecting valve to let a small amount of heavy solution through to the mixing chamber, clearing the valve of air.

5. Fill the mixing chamber with light solution. Add 4 µl TEMED per 12 ml acrylamide solution to each chamber and mix with a disposable pipet.

Form the gradient and cast the gels

6. Turn on the magnetic stirrer. Open the interconnecting valve and allow the chambers to equilibrate. Then slowly open the outlet port to allow the solution to flow from the gradient maker to the multiple caster by gravity (a peristaltic pump may be used for better control). Adjust the flow rate to 3 to 4 ml/min.

   Faster flow rates are possible and will also produce good gradients. However, a fast flow increases the potential for introduction of bubbles into the caster.

7. Close the outlet port as the last of the gradient solution leaves the mixing chamber, just before air enters the outlet tube. Fill the two chambers with plug solution and slowly open the outlet once again.

8. Allow the plug solution to push the acrylamide in the caster up into the plates. Close the outlet when the plug solution reaches the bottom of the plates.

   A discontinuity between the bottom of the gels and the plug solution will be obvious.

9. Quickly add 100 µl H_2O-saturated isobutyl alcohol to each gel sandwich. Let the gels polymerize undisturbed for ~1 hr.

10. Prepare and pour the stacking gel (see Basic Protocol 2, steps 9 and 10).

Disassemble the system

11. Disconnect the gradient maker, place the caster in a sink, and remove the front faceplate. The plug solution will drain out from the bottom of the caster.

12. Remove the gels (see Basic Protocol 2, step 11).

Gradient minigels can be stored as described for single-concentration minigels (see Basic Protocol 2, step 11 annotation). For instructions on preparing, loading, and running the gels, see Basic Protocol 2, steps 12 to 17.

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Anode buffer

121.1 g Tris base (0.2 M final)
500 ml H_2O
Adjust to pH 8.9 with concentrated HCl
Dilute to 5 liters with H_2O
Store at 4°C up to 1 month

Final concentration is 0.2 M Tris·Cl, pH 8.9.

Cathode buffer

12.11 g Tris base (0.1 M final)
17.92 g tricine (0.1 M final)
1 g SDS [0.1% final; recrystallization (see recipe) optional]
Dilute to 1 liter with H_2O
Do not adjust pH
Store at 4°C up to 1 month

Coomassie blue G-250 staining solution

200 ml acetic acid (20% final)
1800 ml H_2O
0.5 g Coomassie blue G-250 (0.025% final)
Mix 1 hr and filter (Whatman no. 1 paper)
Store at room temperature indefinitely

Phosphate/SDS electrophoresis buffer

Dilute 500 ml of 4× phosphate/SDS, pH 7.2 (Table 10.2A.7) with H_2O to 2 liters.
Store at 4°C up to 1 month.

Final concentrations are 0.1 M sodium phosphate (pH 7.2)/0.1% (w/v) SDS.

Phosphate/SDS sample buffer, 2× (for continuous systems)

0.5 ml 4× phosphate/SDS, pH 7.2 (Table 10.2A.7; 20 mM sodium phosphate final)
0.2 g SDS [2% final; recrystallization (see recipe) optional]
0.1 mg bromphenol blue (0.001% final)
0.31 g DTT (0.2 M final)
2.0 ml glycerol (2% final)
Add H_2O to 10 ml and mix

Plug solution

0.125 M Tris·Cl, pH 8.8 (*APPENDIX 2*)
50% (w/v) sucrose
0.001% (w/v) bromphenol blue
Store at 4°C up to 1 month

Recrystallized SDS (optional)

High-purity SDS is available from several suppliers, but for some sensitive applications (e.g., protein sequencing) recrystallization is useful. Commercially available electrophoresis-grade SDS is usually of sufficient purity for most applications.

Add 100 g SDS to 450 ml ethanol and heat to 55°C. While stirring, gradually add 50 to 75 ml hot H_2O until all SDS dissolves. Add 10 g activated charcoal (Norit 1, Sigma) to solution. After 10 min, filter solution through Whatman no. 5 paper on a Buchner funnel to remove charcoal. Chill filtrate 24 hr at 4°C and 24 hr at –20°C. Collect crystalline SDS on a coarse-frit (porosity A) sintered-glass funnel and wash with 800 ml –20°C ethanol (reagent grade). Repeat crystallization without adding activated charcoal. Dry recrystallized SDS under vacuum overnight at room temperature. Store in a desiccator over phosphorous pentoxide (P_2O_5) in a dark bottle.

If proteins will be electroeluted or electroblotted for protein sequence analysis, it may be desirable to crystallize the SDS twice from ethanol/H_2O (Hunkapiller et al., 1983).

SDS electrophoresis buffer, 5×

15.1 g Tris base (0.125 M final)
72.0 g glycine (0.96 M final)
5.0 g SDS [0.5% final; recrystallization (see recipe) optional]
H_2O to 1000 ml
Dilute to 1× or 2× for working solution, as appropriate

Do not adjust the pH of the stock solution, as the solution is pH 8.3 when diluted. Store at 0° to 4°C until use (up to 1 month).

SDS sample buffer, 2× (for discontinuous systems)

25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 10.2A.1)
20 ml glycerol (20% final)
4 g SDS [4% final; recrystallization (see recipe) optional]
2 ml 2-ME *or* 3.1 g DTT (0.2% 2-ME or 0.2 M DTT final)
1 mg bromphenol blue (0.001% final)
Add H_2O to 100 ml and mix
Store in 1-ml aliquots at –70°C

To avoid reducing proteins to subunits (if desired), omit 2-ME or DTT (reducing agent) and add 10 mM iodoacetamide to prevent disulfide interchange.

SDS sample buffer, 6× (for discontinuous systems)

7 ml 4× Tris·Cl/SDS, pH 6.8 (Table 10.2A.1)
3.0 ml glycerol (30% final)
1 g SDS [10% final; recrystallization (see recipe) optional]
0.93 g DTT (0.6 M final)
1.2 mg bromphenol blue (0.012% final)
Add H_2O to 10 ml (if needed)
Store in 0.5-ml aliquots at –70°C

Tricine sample buffer, 2×

2 ml 4× Tris·Cl/SDS, pH 6.8 (Table 10.2A.1; 0.1 M)
2.4 ml (3.0 g) glycerol (24% final)
0.8 g SDS [8% final; recrystallization (see recipe) optional]
0.31 g DTT (0.2 M final)
2 mg Coomassie blue G-250 (0.02% final)
Add H_2O to 10 ml and mix

Background Information

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by *N,N'*-methylenebisacrylamide. The polymerization reaction is initiated by the addition of ammonium persulfate, and the reaction is accelerated by TEMED, which catalyzes the formation of free radicals from ammonium persulfate. Because oxygen inhibits the polymerization process, deaerating the gel solution before the polymerization catalysts are added will speed up polymerization; deaeration is not recommended for the gradient gel protocols because slower polymerization facilitates casting of gradient gels.

Precast gels for commonly used vertical minigel and standard-sized SDS-PAGE apparatuses are available from several manufacturers (Table 10.2A.3). Flatbed (horizontal) isoelectric focusing (IEF) and SDS-PAGE gels are not listed. Amersham Pharmacia Biotech supplies a range of horizontal gels for a variety of applications and should be consulted for further information. When using precast gels, pay strict attention to shelf life. In general, manufacturers overrate the shelf life, and the sooner the gels are used, the better. When reasonably fresh, precast gels provide excellent resolution that is as good or better than a typical gel cast in the laboratory.

The most widely used method for discontinuous gel electrophoresis is the system described by Laemmli (1970). This is the denaturing (SDS) discontinuous method used in Basic Protocol 1. A discontinuous buffer system uses buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel. Because the discontinuous gel system concentrates the proteins in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

In the discontinuous system the sample first passes through a stacking gel, which has large pores. The stacking gel buffer contains chloride ions (called the leading ions) whose electrophoretic mobility is greater than the mobility of the proteins in the sample. The electrophoresis buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility is less than the mobility of the proteins in the sample. The net result is that the faster migrating ions leave a zone of lower conductivity between themselves and the migrating protein. The higher voltage gradient in this zone allows the protcins to move faster and to "stack" in the zone between the leading and trailing ions. After leaving the stacking gel, the protein enters the separating gel. The separating gel has a smaller pore size, a higher salt concentration, and higher pH compared to the stacking gel. In the separating gel, the glycine ions migrate past the proteins, and the proteins are separated according to either molecular size in a denaturing gel (containing SDS) or molecular shape, size, and charge in a nondenaturing gel.

Proteins are denatured by heating in the presence of a low-molecular-weight thiol (2-ME or DTT) and SDS. Most proteins bind SDS in a constant-weight ratio, leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size, not charge. Most proteins are resolved on polyacrylamide gels containing from 5% to 15% acrylamide and 0.2% to 0.5% bisacrylamide (see Table 10.2A.1). The relationship between the relative mobility and log molecular weight is linear over these ranges (Fig. 10.2A.5). With the use of plots like those shown in Figure 10.2A.5, the molecular weight of an unknown protein (or its subunits) may be determined by comparison with known protein standards (Table 10.2A.2). In general, all of the procedures in this unit are suitable for radiolabeled and biotinylated proteins without modification.

Basic Protocol 1 relies on denaturing proteins in the presence of SDS and 2-ME or DTT. Under these conditions, the subunits of proteins are dissociated and their biological activities are lost. A true estimate of a protein's molecular size can be made by comparing the relative mobility of the unknown protein to proteins in a calibration mixture (Fig. 10.2A.5). Gradient gels (Alternate Protocol 5) simplify molecular-weight determinations by producing a linear relationship between log molecular weight of the protein and log % T over a much wider size range than single-concentration gels. Although percent acrylamide monomer is a more common measure of gel concentration, % T, the percentage of total monomer (acrylamide plus bisacrylamide) in the solution or gel, is used for molecular weight calculations in gradient gels. The % T of a stained protein is estimated assuming the acrylamide gradient is linear. For example, proteins in the gel shown in Figure 10.2A.6 were separated in a 5.1% to 20.5% T acrylamide gradient. The % T of the point

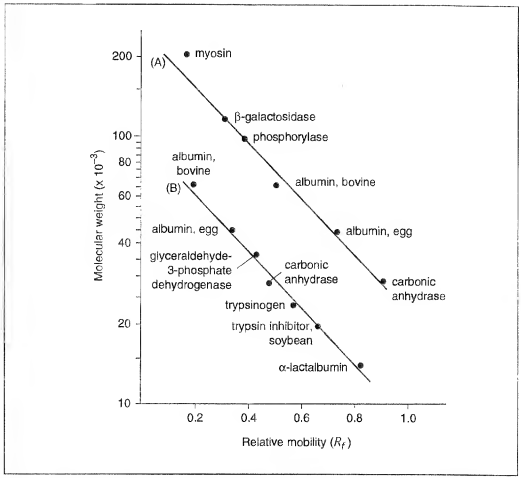

Figure 10.2A.5 Typical calibration curves obtained with standard proteins separated by nongradient denaturing (SDS) discontinuous gel electrophoresis based on the method of Laemmli (1970). (**A**) Gel with 7% polyacrylamide. (**B**) Gel with 11% polyacrylamide. (Redrawn with permission from Sigma.)

halfway through the resolving gel is 12.5% T. Simply plotting log molecular mass versus distance moved into the gel (or R_f) also produces a relatively linear standard curve over a fairly wide size range.

If two proteins have identical molecular sizes, they more than likely will not be resolved with one-dimensional SDS-PAGE, and two-dimensional SDS-PAGE should be considered. Unusual protein compositions can cause anomalous mobilities during electrophoresis (see Critical Parameters and Troubleshooting), but similar-sized proteins of widely different amino acid composition or structure may still be resolved from one another using one-dimensional SDS-PAGE. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will be resolved into constituent polypeptides. Comparison of the protein bands obtained under nonreducing and reducing conditions provides information about the molecular size of disulfide cross-linked component subunits. The individual polypeptides can be isolated by electroelution or electroblotting, and the amino acid sequences can be determined.

Both the tricine (Schagger and von Jagow, 1987) and the modified Laemmli (Okajima et al., 1993) peptide separation procedures presented here (Alternate Protocols 1 and 2) are simple to set up and provide resolution down to 5 kDa. To separate peptides below 5 kDa, the tricine procedure must be modified by preparing a 16.5% T, 2.7% C resolving gel that uses a 10% T spacer gel between the stacking and resolving gel (Schagger and von Jagow, 1987). % C is the percentage of cross-linker (bisacrylamide) in the total monomer (acrylamide plus bisacrylamide).

Continuous electrophoresis, where the same buffer is used throughout the tank and gel, is popular because of its versatility and simplicity. The phosphate system described in Alternate Protocol 3 is based on that of Weber et al.

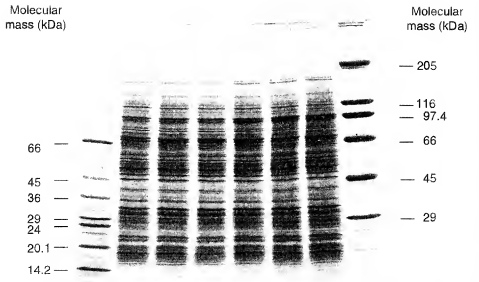

Figure 10.2A.6 Separation of membrane proteins by 5.1% to 20.5% T polyacrylamide gradient SDS-PAGE. Approximately 30 µl of 1× SDS sample buffer containing 30 µg of Alaskan pea (*Pisum sativum*) membrane proteins was loaded in wells of a 14 × 14–cm, 0.75-mm-thick gel. Standard proteins were included in the outside lanes. The gel was run at 4 mA for ~15 hr.

(1972). Although unable to produce the high-resolution separations of the discontinuous SDS-PAGE procedures, continuous SDS-PAGE uses fewer solutions with one basic buffer and no stacking gel. Artifacts are also less likely to occur in continuous systems. Pepsin, for example, migrates anomalously on Laemmli-based discontinuous SDS-PAGE but has the expected mobility after electrophoresis in the phosphate-based continuous system described here. This is also true of cross-linked proteins.

Multiple gel casting (Support Protocols 1 to 3) is appropriate when gel-to-gel consistency is paramount or when the number of gels processed exceeds five a week. The variety of multiple gel casters, gradient makers, and inexpensive pumps available from major suppliers simplifies the process of casting gels in the laboratory. Alternatively, precast gradient gels are available for most major brands of gel apparatuses (Table 10.2A.3).

Minigels (Basic Protocol 2) are generally considered to be in the 8 × 10–cm size range, although there is considerable variation in exact size. Every technique that is used on larger systems can be translated with little difficulty into the minigel format. This includes standard and gradient SDS-PAGE and separations for immunoblotting and peptide sequencing. Two-dimensional SDS-PAGE electrophoresis also adapts well, but here the limitation of separation area becomes apparent; for high-resolution separations, large-format gels are required. Gradient minigels (Support Protocol 3) are popular due to the combination of separation range and resolution (Matsudaira and Burgess, 1978). They are particularly useful for separation of proteins prior to peptide sequencing.

Mylar support (Gel Bond) provides a practical way of casting, running, and, staining extremely thin gels. When gels <0.75 mm thick are used, reagents have much better access both into and out of the gel, reducing staining time in both Coomassie blue and silver staining. Double and broadened images caused by differential migration of the protein across the thickness of the gel are minimized, improving resolution.

Critical Parameters and Troubleshooting

If an electrophoretically separated protein will be electroeluted or electroblotted for sequence analysis, the highest-purity reagents available should be used. If necessary, SDS can be purified by recrystallization following the procedure given in Reagents and Solutions.

If the gels polymerize too fast, the amount of ammonium persulfate should be reduced by one-third to one-half. If the gels polymerize too slowly or fail to polymerize all the way to the top, use fresh ammonium persulfate or increase the amount of ammonium persulfate by one-

third to one-half. The overlay should be added slowly down the spacer edge to prevent the overlay solution from crashing down and disturbing the gel interface.

After a separating gel is poured, it may be stored with an overlay of the same buffer used in the gel. Immediately prior to use, the stacking gel should be poured; otherwise, there will be a gradual diffusion-driven mixing of buffers between the two gels, which will cause a loss of resolution.

The protein of interest should be present in 0.2- to 1-μg amounts in a complex mixture of proteins if the gel will be stained by Coomassie blue (*UNIT 10.6*). Typically, 30 to 50 μg of a complex protein mixture in a total volume of <20 μl is loaded on a 0.75-mm-thick slab gel (16 cm, 10 wells).

When casting multiple gradient gels, eliminate all bubbles in the outlet tubing of the gradient maker. If air bubbles get into the outlet tube, they may flow into the caster and then up through the gradient being poured, causing an area of distortion in the polymerized gel. Air bubbles are not so great a problem when casting single gradient gels from the top. As the gels are cast, the stirrer must be slowed so that the vortex in the mixing chamber does not allow air to enter the outlet.

Uneven heating of the gel causes differential migration of proteins, with the outer lanes moving more slowly than the center lanes (called smiling). Increased heat transfer eliminates smiling and can be achieved by filling the lower buffer chamber with buffer all the way to the level of the sample wells, by maintaining a constant temperature between 10° to 20°C, and by stirring the lower buffer with a magnetic stirrer. Alternatively, decrease the heat load by running at a lower current.

If the tracking dye band is diffuse, prepare fresh buffer and acrylamide monomer stocks. If the protein bands are diffuse, increase the current by 25% to 50% to complete the run more quickly and minimize band diffusion, use a higher percentage of acrylamide, or try a gradient gel. Lengthy separations using gradient gels generally produce good results (Fig. 10.2A.6). Check for possible proteolytic degradation that may cause loss of high-molecular-weight bands and create a smeared banding pattern.

If there is vertical streaking of protein bands, decrease the amount of sample loaded on the gel, further purify the protein of interest to reduce the amount of contaminating protein applied to the gel, or reduce the current by 25%. Another cause of vertical streaking of protein bands is precipitation, which can sometimes be eliminated by centrifuging the sample or by reducing the percentage of acrylamide in the gel.

Proteins can migrate faster or slower than their actual molecular weight would indicate. Abnormal migration is usually associated with a high proportion of basic or charged amino acids (Takano et al., 1988). Other problems can occur during isolation and preparation of the protein sample for electrophoresis. Proteolysis of proteins during cell fractionation by endogenous proteases can cause subtle band splitting and smearing in the resulting electrophoretogram (electrophoresis pattern). Many endogenous proteases are very active in SDS sample buffers and will rapidly degrade the sample; thus, first heating the samples to 70° to 100°C for 3 min is recommended.

In some cases, heating to 100°C in sample buffer will cause selective aggregation of proteins, creating a smeared layer of Coomassie blue–stained material at the top of the gel (Gallagher and Leonard, 1987). To avoid heating artifacts and also prevent proteolysis, the use of specific protease inhibitors during protein isolation and/or lower heating temperatures (70° to 80°C) have been effective (Dhugga et al., 1988).

Although continuous gels suffer from poor band sharpness, they are less prone to artifacts caused by aggregation and protein cross-linking. If streaking or aggregation appear to be a problem with the Laemmli system, then the same sample should be subjected to continuous SDS-PAGE to see if the problem is intrinsic to the Laemmli gel or the sample.

If the protein bands spread laterally from gel lanes, the time between applying the sample and running the gel should be reduced in order to decrease the diffusion of sample out of the wells. Alternatively, the acrylamide percentage should be increased in the stacking gels from 4% to 4.5% or 5% acrylamide, or the operating current should be increased by 25% to decrease diffusion in the stacking gel. Use caution when adding 1× SDS electrophoresis buffer to the upper buffer chamber. Samples can get swept into adjacent wells and onto the top of the well arm.

If the protein bands are uneven, the stacking gel may not have been adequately polymerized. This can be corrected by deaerating the stacking gel solution thoroughly or by increasing the ammonium persulfate and TEMED concentrations by one-third to one-half. Another cause

of distorted bands is salt in the protein sample, which can be removed by dialysis, gel filtration, or precipitation. Skewed protein bands can be caused by an uneven interface between the stacking and separating gels, which can be corrected by starting over and being careful not to disturb the separating gel while overlaying with isobutyl alcohol.

If a run takes too long, the buffers may be too concentrated or the operating current too low. If the run is too short, the buffers may be too dilute or the operating current too high.

If double bands are observed, the protein may be partially oxidized or partially degraded. Oxidation can be minimized by increasing the 2-ME concentration in the sample buffer or by preparing a fresh protein sample. If fewer bands than expected are observed and there is a heavy protein band at the dye front, increase the acrylamide percentage in the gel.

Anticipated Results

Polyacrylamide gel electrophoresis done under denaturing and reducing conditions should resolve any two proteins, except two of identical size. Resolution of proteins in the presence of SDS is a function of gel concentration and the size of the proteins being separated. Under nondenaturing conditions, the biological activity of a protein will be maintained.

Comparison of reducing and nonreducing denaturing gels can also provide valuable information about the number of disulfide cross-linked subunits in a protein complex. If the subunits are held together by disulfide linkages, the protein will separate in denaturing gels as a complex or as smaller-sized subunits under nonreducing or reducing conditions, respectively. However, proteins separated on nonreducing denaturing gels appear more diffuse and exhibit less overall resolution than those separated on reducing gels.

Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins, making them ideal for most types of experiments in spite of being more difficult to prepare. Molecular-weight calculations are simplified because of the extended linear relationship between size and protein position in the gel. Increased band sharpness of both high- and low-molecular-weight proteins on the same gel greatly simplifies survey experiments, such as gene expression studies where the characteristics of the responsive protein are not known. Furthermore, the increased resolution dramatically improves autoradiographic analysis. Preparation of gradient gels is straightforward, although practice with gradient solutions containing dye is recommended. The gradient gels can be stored for several days at 0° to 4°C before casting the stacking gel.

Time Considerations

Preparation of separating and stacking gels requires 2 to 3 hr. Gradient gels generally take 5 min to cast singly. Casting multiple single-concentration gels requires an additional 10 min for assembly. Casting multiple gradient gels takes 15 to 20 min plus assembly time. It takes 4 to 5 hr to run a 14 × 14–cm, 0.75-mm gel at 15 mA (70 to 150 V), and 3 to 4 hr to run a 0.75-mm gel at 20 mA (80 to 200 V). Overnight separations of ~12 hr require 4 mA per 0.75-mm gel. It takes 4 to 5 hr to run a 1.5-mm gel at 30 mA. Electrophoresis is normally performed at 15° to 20°C, with the temperature held constant using a circulating water bath. For air-cooled electrophoresis units, lower currents and thus longer run times are recommended.

It takes ~1 hr to run a 0.75-mm minigel at 20 mA (100 to 120 V). Separation times are not significantly different for gradient minigels.

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Key Reference

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An excellent book describing gel electrophoresis of proteins.

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